Induction and Characterization of *Ph1* Wheat Mutants

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ABSTRACT

The cloning of genes for complex traits in polyploid plants that possess large genomes, such as hexaploid wheat, requires an efficient strategy. We present here one such strategy focusing on the homeologous pairing suppressor (*Ph1*) locus of wheat. This locus has been shown to affect both premeiotic and meiotic processes, possibly suggesting a complex control. The strategy combined the identification of lines carrying specific deletions using multiplex PCR screening of fast-neutron irradiated wheat populations with the approach of physically mapping the region in the rice genome equivalent to the deletion to reveal its gene content. As a result, we have located the *Ph1* factor controlling the euploid-like level of homologous chromosome pairing to the region between two loci (*Xrgc846* and *Xpsr150A*). These loci are located within 400 kb of each other in the rice genome. By sequencing this region of the rice genome, it should now be possible to define the nature of this factor.

HEXAPLOID wheat (*Triticum aestivum* L., $2n = 42$) is composed of three related genomes (A, B, and D), each consisting of seven pairs of chromosomes (Sears 1952). Although each chromosome of hexaploid wheat has the potential to pair with either its homologue or the two pairs of homeologues from the other two genomes, chromosome pairing is largely restricted to homologues. Thus, hexaploid wheat behaves meiotically as a diploid with 21 bivalents observed at meiotic metaphase I. The major locus, *Ph1*, controlling this behavior is located on chromosome *5B* (Okamoto 1957; Riley and Chapman 1958). Two deletions of this locus have long been known, one in hexaploid wheat (*ph1b*; Sears 1977) and the other in tetraploid (A and B genomes) wheat (*ph1c*; Giorgi 1978). Association and recombination between the homeologous chromosomes of the related genomes is evident in these deletion lines as observed by multivalent formation at meiotic metaphase I. Over the last 20 years, the two deletion lines have been important for breeding purposes because they permit the wheat chromosomes to pair with homeologues from related species.

In the past, the absence of the *Ph1* locus has been defined by the presence of multivalents at meiotic metaphase I. Recent results indicate that the deletion of the *Ph1* locus may affect several premeiotic and meiotic processes. In summary, it affects the level of premeiotic association of homologues at their centromeres (Aragon-Alcaide *et al.* 1997a; Martinez-Perez *et al.* 1999), centromere structure (Aragon-Alcaide *et al.* 1997b), the timing of telomere bouquet formation during meiotic prophase and, hence, the timing of intimate association of homologues (Martinez-Perez *et al.* 1999), and recombination between homeologous chromosomes or segments (Dubcovsky *et al.* 1995; Luo *et al.* 1996). Sears (1977) also reported that the *ph1b* line exhibited reduced fertility compared to euploid wheat, implying chromosome pairing/segregation failure and gamete abortion. Recent studies suggest some form of premeiotic control of chromosome pairing in wheat (Aragon-Alcaide *et al.* 1997a; Vega and Feldman 1998). However, from our current knowledge of chromosome pairing, largely based on yeast studies, it is difficult to envisage how all the effects observed might be controlled by a single gene, although it is possible that the disruption of a premeiotic process could result in a cascade of effects on meiotic processes. It is therefore important to establish whether the *Ph1* locus is a single gene, a multigene family, or a complex of linked genes influencing different meiotic processes.

The deleted segment of chromosome *5B* in the *ph1b* line is ~70 Mb in size and, therefore, a number of genes are likely to have been deleted (Gill *et al.* 1993, 1996). Further analysis of the genetic content of this locus requires identification of new deletions involving the region and an approach for revealing the gene content of this 70-Mb region of the wheat genome. A strategy has been suggested for using rice as the cereal model (Moore *et* al. 1993). The genome of hexaploid wheat consists of 17,000 Mb per 1C nucleus (Bennett and Smith 1991) and is composed of ~80% repetitive sequences. The rice genome on the other hand is only 400 Mb and is composed of only 50% repetitive sequences. Comparative mapping has revealed that the genes on

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rice chromosome *9* are in a similar order to those on a part of wheat chromosome *5B* including the region containing the *Ph1* locus (Foote *et al.* 1997). The present study describes an approach of combining the identification and characterization of wheat lines carrying new deletions of the *Ph1* locus with the establishment of a complete yeast artificial chromosome (YAC) contig to the corresponding region in rice.

MATERIALS AND METHODS

Plant material: The following hexaploid wheat (*T. aestivum*; $2n = 6x = 42$; genome AABBDD) lines were used: euploid Chinese Spring; the Chinese Spring mutant line *ph1b* (Sears 1977); Chinese Spring nullisomic-tetrasomic lines N*5A*T*5B*, N*5B*T*5D*, and N*5D*T*5A* (Sears 1966); and a doubled haploid single chromosome substitution line, Highbury (CS*5B*) line in which the Highbury *5B* chromosome has been substituted by that from Chinese Spring (Snape *et al.* 1988). The tetraploid wheat (*T. durum*; $2n = 4x = 28$; genome AABB) Cappelli and its derivative deletion line *ph1c* (Giorgi 1978) were used in deletion mapping experiments. Deletion lines for screening were generated *de novo* in two separate experiments. First, 3000 seeds from the wheat line Highbury (CS*5B*) were subjected to a fast-neutron irradiation dose of 3.0 GyNf at the International Atomic Energy Agency's Laboratories (Seibersdorf, Austria; protocol no. FN662). The M_1 generation was grown in a greenhouse, allowed to self-fertilize, and the seed was harvested. A total of 5213 seeds from 1042 M_1 lines were sown in field plots with up to 15 plants per row (each row representing one M_1 line), and progeny from 25 M_1 different lines were selected for further analysis because they exhibited reduced fertility in mature spikelets. The M_3 generation from the 25 selected M_1 lines was grown under greenhouse conditions and screened for deletions of the *Ph1* locus by PCR (Qu *et al.* 1998). M_4 plants from one M_1 line, H455, were analyzed further.

For the second experiment, 1300 F_1 seeds from *ph1b* segmental hemizygotes of a cross between Chinese Spring and the *ph1b* deletion line were subjected to fast-neutron irradiation (as above). Half of the M_1 seeds were grown in a greenhouse and selfed seed was collected. Almost 5000 juvenile M_2 plants (up to 10 from each M_1 parent) were screened using the multiplex PCR *ph1b* assay, and selected plants were selfed or crossed with Chinese Spring lines and grown further.

PCR-based characterization of the *ph1b* domain: Primers PSR2120.f (5′-TTA ACG CCA GGG CAT ACT C) and PSR2120.r (5′-CTG CAG GAG GCG CTG GA) amplify a wheat 232-bp DNA fragment PC3-9 (subsequently designated *Xpsr2120*) that maps within the *ph1b* deletion and can be used in a plus/minus PCR assay to detect lines deleted in this region (Qu *et al.* 1998). The DNA sequences of two restriction fragment length polymorphism (RFLP) probes PSR128 and PSR574 were known (J. B. Smith, K. M. Devos and M. D. Gale, personal communication) that have been located in the *ph1b* deletion (Dunford *et al.* 1995). Therefore, to obtain additional PCR-based markers, primers were designed to amplify B genome-specific DNA fragments of these two probes. Optimal primer pairs for B genome-specific *Xpsr128* and *Xpsr574* amplification were designated PSR128.f (5′-ATC GCT CCT CTG CTT GCT TC) and PSR128.r (5′-GAC CGC CTG AAA CCT CCC), and PSR574.f (5′-AGC GTA TAT TCA CGC GCT CC) and PSR574.r (5′-CGT AAG AAC TCC CCA GGG TTT G), respectively. Primers AWJL3.f (5′-TGG CAC CCT CAA TGT AGA C) and AWJL3.r (5′-GCT TGC CCA TTT CAC AAC), derived from the early meiosis wheat cDNA clone pAWJL3 (Ji and Langridge 1994) and mapped to wheat chromosome *2A* (M. A. Roberts, unpublished results), were included in multiplex PCR assays as a positive control.

Fresh intact leaf material was prepared for PCR amplification as described by Qu *et al.* (1998). Each 50-μl PCR reaction contained one prepared leaf slice (0.5 cm^2), 1× reaction buffer (10 mm Tris-HCl, pH 9.0, 1.5 mm $MgCl_2$, and 50 mm KCl), 2 units of *Taq* DNA polymerase (Pharmacia Biotech, Piscataway, NJ), 2 mm dNTPs, 50 pmol each primer, and 1× loading buffer (2% Ficoll, 0.1 mm EDTA, and 0.02% Orange G dye). The first PCR reaction cycle contained a denaturating step at 94° for 30 sec, an annealing step at 65° for 30 sec, followed by a product extension step at 72° for 1 min. For the next 12 cycles, the annealing temperature was reduced by 0.7° per cycle, with the final annealing temperature of 56° continued for another 23 cycles (Vos *et al.* 1995). Reaction products were fractionated through 3% agarose gels.

RFLP analysis: Genomic DNA was extracted from wheat lines (Qu *et al.* 1998) and analyzed using standard procedures (Foote *et al.* 1997). When hybridizing with either wheat or rice probes, Southern blots of wheat-digested genomic DNA were blocked with 5 μg/ml (final concentration) denatured sonicated wheat genomic DNA to produce optimal hybridization results.

Cytogenetic analysis: Chromosome pairing of spring sown control and deletion lines was analyzed in pollen mother cells at meiotic metaphase I in Feulgen-stained preparations. Observed differences in pairing frequencies between lines were assessed for statistical significance using Tukey's comparisons for single factor one-way analysis of variation (ANOVA). The single variable analyzed was that of the nature of the chromosome pairing in pollen mother cells derived from the single M_2 and M_4 deletion lines.

YAC contig development: Forty-seven rice YACs located in the rice chromosome *9* region analogous to the overlapping wheat *ph1b* and *ph1c* deletions were selected from our rice chromosome *9* partial YAC physical map (Foote *et al.* 1997) for further analysis. Each of the rice YAC clones was plated out from −70° glycerol stocks onto fresh YPDX complete medium (1% yeast extract, 2% peptone, 2% dextrose, 2% agar, 50 μg/ml uracil, 50 μg/ml l-tryptophan, and 30 μg/ml adenine hemisulfate) and then transferred onto SD minimal medium (0.67% yeast nitrogen base without amino acids, 2% d-glucose, and 2% agar) supplemented with 20 μg/ml adenine hemisulfate, 20 μg/ml l-histidine, 30 μg/ml l-isoleucine and 30 μg/ml l-lysine, to select yeast strain AB1380 colonies containing functional left (*TRP1*) and right (*URA3*) arms of the pYAC4 vector (Burke *et al.* 1987). At least five separate colonies from each YAC clone were picked for high molecular weight mini-prep DNA isolation (adapted from Schwartz and Cantor 1984). DNA was fractionated by pulsed-field gel electrophoresis (PFGE) using the Pharmacia LBK 2015 Pulsaphor System, typically with a 50- to 120-sec pulse at 170 V over 48 hr to separate fragments of 100–800 kb. Following Southern transfer to Hybond N^+ nylon filters, YACs were sized by probing with the 1.4-kb *Pvu*II/*Sal*I pBR322 fragment (pYAC4 right arm probe) and the 2.3-kb *Pvu*II/*Eco*RI pBR322 fragment (pYAC4 left arm probe). Integrity of each YAC clone was assessed by comparing YAC sizes from five or more independent colonies and, if fragmentation or deletion was evident, then the largest YAC clone was used further.

For isolation of YAC ends using the inverse PCR method (Silverman 1993), individual YACs were obtained after PFGE of high-concentration total DNA plugs. YAC DNA was digested with each of the restriction endonucleases *Acc*I, *Eco*RV, *Hae*III, *Hha*I, *Hin*cII, *Nla*III, *Nla*IV, *Rsa*I, *Sal*I, and *Sph*I. DNA fragments were circularized by ligation and subjected to PCR using the following primer pairs with the indicated enzyme combinations for each arm: A5 (5′-ATA CAA TTG AAA AAG AGA

TTC C) and S8 (5′-GTA GCC AAG TTG GTT TAA GG) with *Nla*III for left arms; A13 (5′-GGA CGG GTG TGG TCG CCA TGA TCG CG) and S8 with *Eco*RV, *Hae*III, *Nla*IV, and *Rsa*I for left arms; S2 (5′-GAC TTG CAA GTT GAA ATA TTT CTT TCA AGC) and A3 (5′-AGT CGA ACG CCC GAT CTC AA) with *Hae*III, *Hha*I, *Nla*III, *Nla*IV, and *Sph*I for right arms; and S11 (5′-AGG AGT CGC ATA AGG GAG AG) and A3 with *Acc*I, *Hinc*II, and *Sal*I for right arms. Selected PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, WI). Putative YAC end clones were hybridized to Hybond N^+ filters gridded with the 47 rice YACs to confirm clone identities and to screen for overlapping YACs. DNA sequencing of YAC end clones was performed using the Thermo Sequenase dye terminator cycle sequencing kit (Amersham, Arlington Heights, IL).

RESULTS AND DISCUSSION

Development of multiplex PCR *ph1b* assay: The wheat *ph1b* deletion generated by Sears (1977) spans ~70 Mb (Gill *et al.* 1993). To screen for novel deletion lines with different breakpoints and thereby define more closely the region containing the *Ph1* locus, it was necessary to develop PCR markers for loci dispersed within the *ph1b* deletion. On the long arm of wheat chromosome *5B*, wheat RFLP loci *Xpsr100* (at 3.2 cM from the centromere) and *XKsu8* (at 22.7 cM from the centromere) broadly delimit the *ph1b* deletion (Gale *et al.* 1995; Foote *et al.* 1997). Three PCR-generated loci already reported are all located distally near the *Xksu8* breakpoint (Gill and Gill 1996; Segal *et al.* 1997; Qu *et al.* 1998), so the proximal RFLP loci *Xpsr128* (at 4 cM) and *Xpsr574* (at 10.9 cM) were selected for development into PCR-based assays. Two sets of primer pairs tested, PSR128.f/PSR128.r and PSR574.f/PSR574.r, were found to amplify the expected wheat chromosome *5B* sequences located within the *ph1b* deletion (Figure 1). In combination with the primers PSR2120.f/PSR2120.r (Qu *et al.* 1998) and positive control primers AWJL3.f/AWJL3.r, it proved feasible to undertake a single multiplex reaction, which provided a plus/minus assay for the determining the presence/absence of these three *ph1b*-linked loci.

The multiplex PCR reaction worked efficiently on both purified genomic wheat DNA and wheat leaf slices prepared for PCR using a simple NaOH lysis/boiling technique (Qu *et al.* 1998; Figure 1, a and b). In our hands, neither the KSU8-derived (Gill and Gill 1996) nor the WPG90-derived (Segal *et al.* 1997) primers amplified efficiently with PCR-prepared leaf slices as template. Both assays require time-consuming DNA extraction and the KSU8-derived assay requires restriction enzyme digestion of the PCR products to detect Chinese Spring euploid *vs. ph1b* polymorphisms. The PCR assay developed here has neither of these requirements and therefore allows higher throughput screening of mutagenized lines. However, the KSU8-derived assay still provides a useful marker for this region.

Identification of Highbury (CS5B) *ph1* deletion lines: When initially describing the *ph1b* deletion line, Sears (1977) noted that its average seed yield was only 34% that of euploid Chinese Spring. This reduction in fecundity was attributed to an interstititial deficiency that included the *Ph1* locus. In the present study, M_2 plants from the progenies of selfed fast-neutron irradiated Highbury (CS*5B*) M_1 lines were screened for reduced seed set in mature spikes. Approximately 2.3% of the lines appeared to be segregating for reduced fertility,

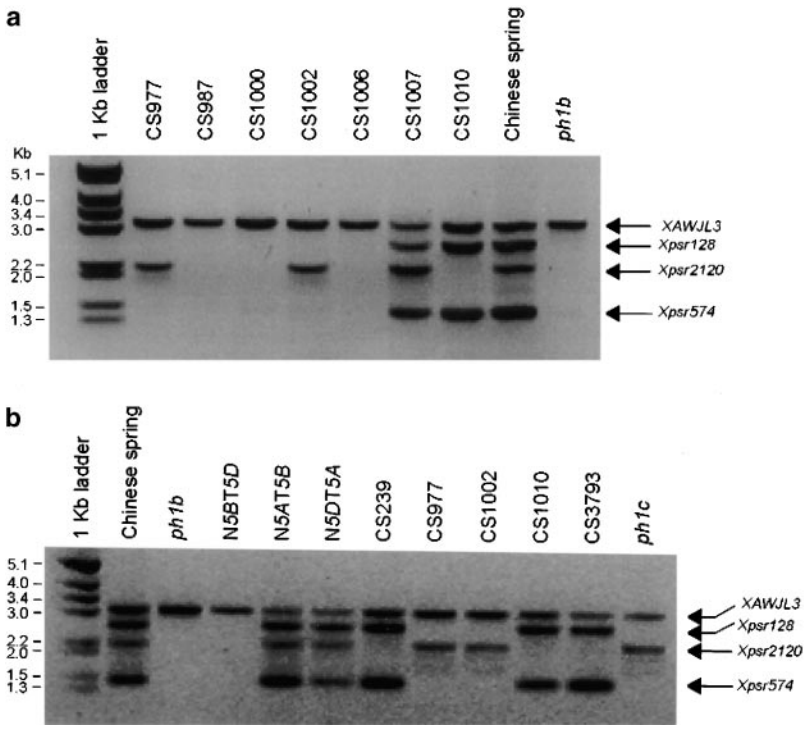

Figure 1.—(a) PCR amplification products from alkaline-treated wheat leaves using primers PSR128f, PSR128r, PSR574f, PSR574r, PSR2120f, PSR2120r, AWJL3f, and AWJL3r multiplexed together (see materials and methods). (b) PCR amplification products from extracted genomic DNA using the same primers multiplexed together. The absence of the product in the nullisomic-tetrasomic line N*5B*T*5D* indicates the mapping of the product to the missing chromosome. Products corresponding to each of the loci are indicated.

and these were selected for further anaylsis. A total of 295 low-fertility segregants (M_2) obtained from the selected self-fertilized 25 M_1 plants were also self-fertilized. Leaf material from 295 M_3 progeny (a single progeny from each M_2 plant) was tested using the PSR2120 PCR-based assay for the *ph1b* deletion (Qu *et al.* 1998). Only 3 M_3 Highbury (CS*5B*) plants, H455-2, H455-9, and H455-11, descended from M_1 line H455, were found to be missing the *ph1b*-located PSR2120 PCR marker. None of the 12 remaining M_3 plants descended from H455, nor any other of the M_3 plants tested, were found to be deficient for *Xpsr2120*. The PSR2120-based PCR assay produced clear results from 269 (91.2%) of the M_3 plants, including 93 (31.5%) that were screened twice, with the balance unscorable due to lack of or unclear PCR amplification products.

The 14 M_3 plants (including H455-2 and H455-9) descended from H455 were allowed to self-fertilize, and M_4 seeds were harvested and germinated. Leaf material from juvenile M_4 plants was screened by the multiplex PCR *ph1b* assay developed in this study for the presence or absence of *Xpsr128*, *Xpsr574*, and *Xpsr2120* loci (Table 1). Two plants of the three M_4 progeny of H455-2, *viz.* H455-2-2 and H455-2-3, were deficient for all three loci. The third plant, H455-2-1, lacked none of the markers and is likely to be the result of spurious out-pollination. H455-9-1, the only progeny of H455-9, lacked only *Xpsr2120*, while H455-11-1, one of the four progeny of H455-11, also lacked the *Xpsr2120* locus. The remaining 43 M_4 plants were not homozygous for deletions within the *ph1b* region.

Southern and PCR analysis of phenol/chloroform-extracted genomic DNA indicated that H455-2-2 was deficient for all markers tested within the *ph1b* region, and therefore the deleted region is of the same size or larger than the *ph1b* deletion (Table 1). H455-2-2 and H455-2-3 possibly carry the same deficiency. However, H455-9-1 was deficient only for loci *Xpsr150A*, *Xpsr2120*, and *Xksu8*, and the plant's interstitial deletion was delimited proximally by *Xrgc846* and distally by *Xpsr150B*. The H455-9-1 deletion is therefore smaller than the *ph1b* deletion. It can be inferred that plants H455-9-1 and H455-11-1 probably possess a similar deficiency. Identification of M_4 plants carrying two different-sized deletions indicates that their common irradiated progenitor line H455 was chimeric.

Identification of Chinese Spring *ph1* deletion lines: In a second experiment to identify novel *Ph1* deficiencies in wheat, seeds hemizygous with respect to the *ph1b* deletion (the F_1 generation from a Chinese Spring euploid × *ph1b*/*ph1b* cross, thus carrying a complete chromosome *5B* and a *ph1b* deletion chromosome *5B*) were irradiated by fast-neutron bombardment. Assuming transmission of both *5B* chromosomes from a *Ph1*/*ph1* heterozygote, three classes of progeny were anticipated from screening M_2 plants with the multiplex PCR *ph1b* assay: (1) all three *ph1b* PCR markers present, in nonde-

TABLE 1

Marker analysis of deletion lines

		Control lines						Highbury lines			CS/*ph1b* hemizygous lines				
5BL loci	Detected by	Euploid CS	*ph1b*	Euploid Cappelli	*ph1c*	N5BT5*D*	(Highbury) CS 5*B*	H455-2-2	H455-9-1	H455-11-1	CS239	CS1010	CS977	CS1002	CS3793
Xpsr128	PCR	+	−	+	−	−	+	−	+	+	+	+	−	−	+
Xpsr574	PCR	+	−	+	−	−	+	−	+	+	+	+	−	−	+
Xrgr2790	RFLP	+	−	+	−	−	+	−	+	+	+	+	−	−	ND
Xrgc846	RFLP	+	−	+	−	−	ND	ND	+	ND	+	+	−	−	ND
Xpsr150A	RFLP	+	−	+	−	−	ND	ND	−	ND	+	−	+	+	ND
Xpsr2120	PCR	+	−	+	+	−	+	−	−	−	−	−	+	+	−
Xksu8	PCR/digestion	+	−	+	+	−	+	−	−	−	−	−	+	+	−
Xpsr150B	RFLP	+	+	+	+	−	ND	ND	+	ND	+	−	+	+	ND

+, marker present; −, marker absent; ND, not determined/unclear.

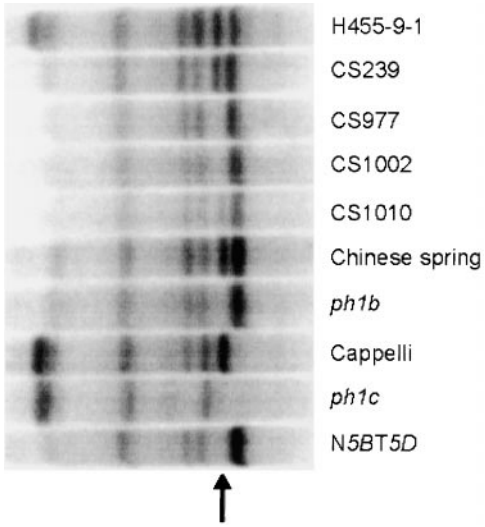

Figure 2.—PhosphorImage of a Southern blot of genomic DNA digested with *Hin*dIII and hybridized with the rice DNA probe R2790. The arrow indicates the band missing in N*5B*T*5D*, *ph1b*, *ph1c*, CS977, and CS1002 lines. The absence of the band in the N*5B*T*5D* indicates the mapping of the band to the missing chromosome *5B*.

leted homozygous *Ph1* plants (expected frequency $F \approx 0.25$) and from hemizygous *ph1b* deletion plants ($F \approx 0.5$); (2) all three *ph1b* PCR markers absent, in homozygous *ph1b* plants ($F \approx 0.25$) and in hemizygous plants with the *ph1b* deletion and novel *5BL* deletions of similar size or completely spanning the *ph1b* region (unknown *F*); and (3) one or two *ph1b* PCR markers absent, in plants homozygous or hemizygous for a novel deficiency within the *ph1b* region (unknown *F*). For the purposes of this experiment, only class 3 progeny were studied further.

Up to five M_2 progeny from each of two ears chosen at random from 450 M_1 plants were screened using the multiplex PCR *ph1b* assay. Five novel deletion lines were identified among the 4283 juvenile plants screened: CS239, CS1010, and CS3793 lacked the *Xpsr2120* PCR locus but possessed *Xpsr128* and *Xpsr574* loci, while CS977 and CS1002 lacked both *Xpsr128* and *Xpsr574* but possessed *Xpsr2120* (Table 1; Figure 1). The multiplex PCR *ph1b* assay generated scorable results in 98.9% of plants tested, including 13.5% that were repeated because the initial results were unclear.

Southern RFLP and PCR analysis of phenol/chloroform-extracted genomic DNA from the novel deletion lines confirmed the results of the leaf-based assays and allowed for further characterization (Table 1; Figure 2). The deficiency in CS239 could be delimited by two loci of the probe PSR150 located on the distal portion of chromosome *5BL*. *Xpsr150A* had been previously located within the *ph1b* and *ph1c* deletions while *Xpsr150B* was located outside both deletions (Foote *et al.* 1997). In contrast, the deficiency in CS1010 was delimited outside these two *Xpsr150* loci, providing another deletion breakpoint within the *ph1b* region. Lines CS977 and CS1002 harbored deficiencies that encompassed the proximal region of the *ph1b* deletion, and both the deficiencies extended distally from the locus *Xrgc846* (identified using a rice probe) up to breakpoints before *Xpsr150A* (Table 1; Figure 2). The deficiency in line CS3793, which lacks the PCR locus *Xpsr2120* located in the distal region of the *ph1b* deletion, also encompasses *Xksu8* (Table 1).

The screening and partial genotyping of deleted M_2 juvenile plants has resulted in the identification of five new lines that contain deficiencies with different breakpoints within the *ph1b* region. It is important to remember, however, that the experiment was designed to allow detection of deficiencies overlapping with the *ph1b* deletion in both homozygous and heterozygous conditions. Only one line, CS1010, deficient for the locus *Xpsr150B* that is present in the *ph1b* line (Table 1), can be identified as homozygous for its deficiency, while the remaining four lines could be either homozygous or *ph1b* hemizygous for their respective deficiencies. It was still feasible to analyze the chromosome pairing behavior of both hemizygous and homozygous M_2 lines to attempt to correlate the different deficiencies with pairing phenotype, because only one copy of the *Ph1* locus is necessary to prevent wheat meiotic homeologous pairing and maintain diploid-like homologous pairing (Okamoto 1957; Riley and Chapman 1958).

Cytological analysis of *ph1* deletion lines: Meiotic pairing in wheat lines is commonly assessed using F_1 hybrids produced from crosses between the study line and related Triticeae species, *e.g.*, *Secale cereale* (rye; Wall *et al.* 1971), *Aegilops variabilis* (Sears 1977; Ceoloni and Donini 1993) and *A. sharonensis* (Mello-Sampayo 1972). In these F_1 hybrids homeologous chromosome associations are clear due to the absence of any homologous chromosome pairs, and the effects of genetic alterations can therefore be more easily diagnosed. However, such F_1 hybrids are sterile, and their production is time consuming. Moreover, interpretation of the effect of the *Ph1* locus can be complicated by the presence of additional pairing loci on related Triticeae chromosomes. Therefore in this study meiotic pairing was only assessed in the control and deletion lines themselves. The feasibility of differentiating Chinese Spring euploid and Chinese Spring N*5B*T*5D* lines using selfed material has been demonstrated, albeit that differences in univalent, bivalent, and higher-order chromosome associations between lines are subtle (Riley and Kempanna 1963).

Highbury (CS *5B*) M_4 deletions lines, Chinese Spring M_2 deletion lines, and appropriate controls were scored in blind trials at meiotic metaphase I for univalent, rod and ring bivalent, and higher-order association (tri-, quadri-, penta-, and hexavalent) chromosome formations (Table 2). The 17 controls contained progeny derived from the same ears as the deletion lines, includ-

TABLE 2

Chromosome pairing at metaphase I of specific mutagenized lines

Line/genotype	Univalents	Bivalents			Higher associations
		Rod	Ring	Total	
CS (*Ph1*/*Ph1*)	0.00 ± 0.00 (0) (a)	1.0 ± 1.10 (0–4) (e)	20 ± 1.10 (17–21) (l)	21 ± 0.00 (21) (r)	0
CS178	0.03 ± 0.18 (0–1) (a)	1.13 ± 1.04 (0–3) (e)	19.5 ± 1.11 (18–21) (l)	20.6 ± 0.81 (19–21) (r)	0.20 ± 0.41 (0–1)
CS1219	0.13 ± 0.15 (0–2) (ab)	1.67 ± 1.12 (0–4) (e)	19.2 ± 1.32 (17–21) (l)	20.9 ± 0.43 (19–21) (r)	0.03 ± 0.18 (0–1)
CS239	0.07 ± 0.25 (0–1) (ab)	1.07 ± 0.98 (0–3) (e)	19.2 ± 1.58 (16–21) (l)	20.3 ± 1.23 (20–21) (qr)	0.37 ± 0.61 (0–2)
CS (*ph1b*/*ph1b*)	0.8 ± 1.12 (0–4) (bc)	4.73 ± 1.41 (1–7) (g)	14.8 ± 1.82 (10–18) (jk)	19.6 ± 1.52 (17–21) (pqr)	0.57 ± 0.77 (0–3)
CS276	0.67 ± 1.06 (0–4) (abc)	4.23 ± 1.79 (0–7) (g)	15.2 ± 1.73 (11–21) (jk)	19.4 ± 1.52 (17–21) (opqr)	0.67 ± 0.76 (0–2)
CS270	0.93 ± 1.39 (0–4) (b)	3.60 ± 1.94 (0–7) (g)	14.7 ± 1.83 (9–18) (jk)	18.3 ± 1.72 (14–21) (nop)	1.13 ± 1.01 (0–3)
CS1002	0.87 ± 0.78 (0–2) (c)	2.10 ± 1.47 (0–5) (ef)	16.0 ± 1.68 (13–19) (k)	18.07 ± 0.94 (17–20) (opq)	1.33 ± 0.55 (0–2)
CS1010	0.83 ± 1.26 (0–4) (abc)	3.57 ± 1.30 (0–6) (efg)	15.4 ± 1.71 (12–19) (k)	18.97 ± 1.03 (17–21) (opq)	0.90 ± 0.66 (0–2)
H455-2-3	1.00 ± 1.08 (0–3) (c)	3.60 ± 1.90 (0–8) (fg)	13.5 ± 2.54 (9–17) (j)	17.1 ± 2.45 (12–21) (n)	1.80 ± 1.21 (0–3)
H455-2-2*	2.56 ± 1.89 (0–4)	4.13 ± 1.63 (2–8)	12.2 ± 2.54 (6–18)	16.3 ± 2.38 (11–20)	1.53 ± 1.14 (0–4)
H455-9-1	5.70 ± 3.92 (1–19) (d)	7.70 ± 1.95 (2–10) (h)	5.87 ± 3.37 (2–13) (i)	13.6 ± 2.56 (8–18) (m)	2.37 ± 0.89 (0–3)
H455-11-1**	5.00	7.00	4.67	11.67	2.67

Values are the mean ± SD. Higher associations are tri-, quadri-, penta-, and hexavalent formation. For each column the same letter denotes that the values are not significantly different ($P = 0.01$) based on Tukey's comparisons for single factor one-way ANOVA. Range of values for each configuration is provided in parentheses. Thirty cells were scored for each line except H455-11-1**, where only 3 were scored and the line perished. All the lines possessed 42 chromosomes except for H455-2-2*, which had 41 chromosomes.

ing those identified as having a *ph1*/*ph1* genotype (*i.e.*, loci *Xpsr2120*, *Xpsr574*, and *Xpsr128* absent). Lines such as CS178 and CS1219 (both $2n = 42$), which appeared from the multiplex PCR *ph1b* assay to be homozygous *Ph1*/*Ph1* or hemizygous *Ph1*/*ph1* (*i.e.*, loci *Xpsr2120*, *Xpsr574*, and *Xpsr128* present), formed predominantly 21 bivalents at MI and exhibited low levels of univalent and higher-order chromosome formation, as expected for Chinese Spring lines carrying the *Ph1* locus. In contrast, lines CS270 and CS276 (also both $2n = 42$), which were diagnosed by PCR as having a *ph1*/*ph1* genotype, formed slightly fewer bivalents and more higher-order chromosome associations, similar in phenotype to Sears' *ph1b*/*ph1b* strain (Table 2). Although the total number of bivalents formed in *Ph1* euploid and *ph1* deficient lines was not statistically distinct ($P = 0.01$), it proved informative enough to differentiate between rod (mainly homeologous) and ring (mainly homologous) bivalent formation: lines CS(*ph1b*/*ph1b*), CS270, and CS276 that are deficient for the *Ph1* locus produced more rod bivalents and less ring bivalents than lines CS178 and CS1219 that contain the *Ph1* locus (each

character statistically significant at $P = 0.01$). Data from cellular and cytogenetical studies suggest that the *Ph1* locus must in part function to actively promote homologous pairing and cannot simply be preventing homeologous pairing (Gillies 1987; Aragon-Alcaide *et al.* 1997a; Martinez-Perez *et al.* 1999). This suggests that the level of homologous chromosome pairing is an appropiate measure for the effect of the *Ph1* locus. Therefore, to assess whether novel deletion lines were normal or abnormal for homologous chromosome pairing behavior, the number of ring bivalents in control and experimental lines was compared.

Three classes of pairing phenotype could be distinguished on the basis of ring bivalent formation during meiotic metaphase I (statistically significant at $P = 0.01$): Chinese Spring euploid frequencies of ring bivalent formation were seen in CS239: H455-2-3, CS1002, and CS1010 produced similar numbers of ring bivalents to the CS(*ph1b*/*ph1b*) deletion, and very low levels of ring bivalent associations were observed in H455-9-1 (Table 2). Line H455-2-2 had only 41 chromosomes and was not included in the statistical analyses; however, it appears to have a similar level of homologous pairing to the CS(*ph1b*/*ph1b*) deletion.

Only three cells could be scored in line H455-11-1 ($2n = 40$), and these limited data indicated that the line had homologous chromosome pairing behavior similar to H455-9-1 (Table 2). However, the line was completely sterile and has perished. The lines CS3793 and CS977 were also not robust. Only eight pollen mother cells could be scored in the M_2 line CS977 ($2n = 42$) and the line had an apparent *ph1b*-like phenotype, with 0.50 ± 0.53 univalents, 3.88 ± 2.10 rod bivalents, and 14.38 ± 1.77 ring bivalents. Extensive backcrossing of these lines is being undertaken to improve their viability. If improved viability is achieved, the pairing frequencies of their progeny will then be assessed. All the lines identified by the screening procedures except for the CS239 (which possessed euploid-like pairing behavior) exhibited reductions in fertility and viability. Lines H455-9-1, CS977, and CS3793 exhibit the most severe levels of sterility. Sears (1977) observed a low transmission rate for the chromosomes carrying the *ph1b* deletion. The transmission rate for chromosomes carrying the new deletions appears even lower than that of the *ph1b* chromosome. This may account for the high levels of sterility observed in these lines.

Irradiation can induce nonreciprocal chromosome translocations resulting in multivalents at metaphase I and apparent "homeologous pairing." The level of multivalent formation did not accurately predict the presence or absence of the *Ph1* locus (Table 2). Its scoring may be complicated by the occurrence of such translocations. The presence of multivalents suggests that both homeologous pairing and recombination has occurred, while the presence of univalents, as Gillies (1987) indicated, could result from homeologous pairing without homeologous recombination. Pollen mother cells derived from the *ph1b* line often do not possess any multivalents but do possess univalents. Therefore it is not surprising that the frequency of multivalents does not accurately predict the absence of the *Ph1* locus. However, by scoring the frequency of ring bivalents, the number of chromosomes engaged in homologous pairing can be estimated. Conversely, the number of chromosomes not present as ring bivalents provides an estimate for those chromosomes engaged in both homeologous pairing with and without homeologous recombination. The use of ring bivalent frequencies at metaphase I in blind scoring of the lines proved sufficiently accurate to identify the lines previously determined by PCR to be homozygous for the *ph1b* deletion, including progeny from the same ear as the deletion lines. If there were nonreciprocal translocations in the M_1 plants, then they did not interfere with the characterization of these M_2 progeny. Therefore it is reasonable to suppose that the determination of the pairing types of the progeny carrying the new *Ph1* deletions was also accurate. For the Highbury (CS *5B*) deletion lines, however, it was not possible to undertake a similar study. Therefore the H455-9-1 line could carry nonreciprocal translocations causing the apparently higher level of homeologous pairing. An alternative explanation is that the deletion breakpoint in this line is actually located within the *Ph1* locus, resulting in a new phenotype.

Characterizing the *Ph1* locus: The cytological analyses have shown the meiotic chromosome pairing behavior of novel wheat lines with deletions within the *ph1b* domain. The data presented in Tables 1 and 2 allow for the construction of a map of deletion breakpoints that minimally define the part of the *Ph1* region on chromosome *5B* that controls the level of homologous/homeologous pairing (Figure 3, top). Deletion line CS1010 is deleted distal to the locus *Xrgc846*, and line CS1002 is deleted proximal to *Xpsr150A*, yet both lines show *ph1b*-like level of homologous pairing behavior. Line CS977 is deleted for the same markers as CS1002, and at the M_2 generation both lines exhibited *ph1b*-like behavior. Therefore, the euploid-like level of homologous chromosome pairing is being controlled by a factor that must lie between loci *Xrgc846* and *Xpsr150A*. Line CS239, deleted distal to locus *Xpsr150A*, exhibits euploid-like pairing, confirming that this factor cannot be located distal to *Xpsr150A*.

Line H455-9-1, with the same *Ph1* marker deficiencies as CS1010 (but possesses the locus *Xpsr150B*, which CS1010 does not), exhibited higher levels of homeologous pairing than lines CS1010, CS1002, CS977 and Sears' *ph1b*/*ph1b* deletion line (Figure 3 and Table 2). As indicated previously, it is possible that the breakpoint of the deletion in H455-9-1 disrupts the *Ph1* factor, causing an abnormal and more pronounced phenotype than complete loss of the gene or genes, or possesses nonreciprocal translocations.

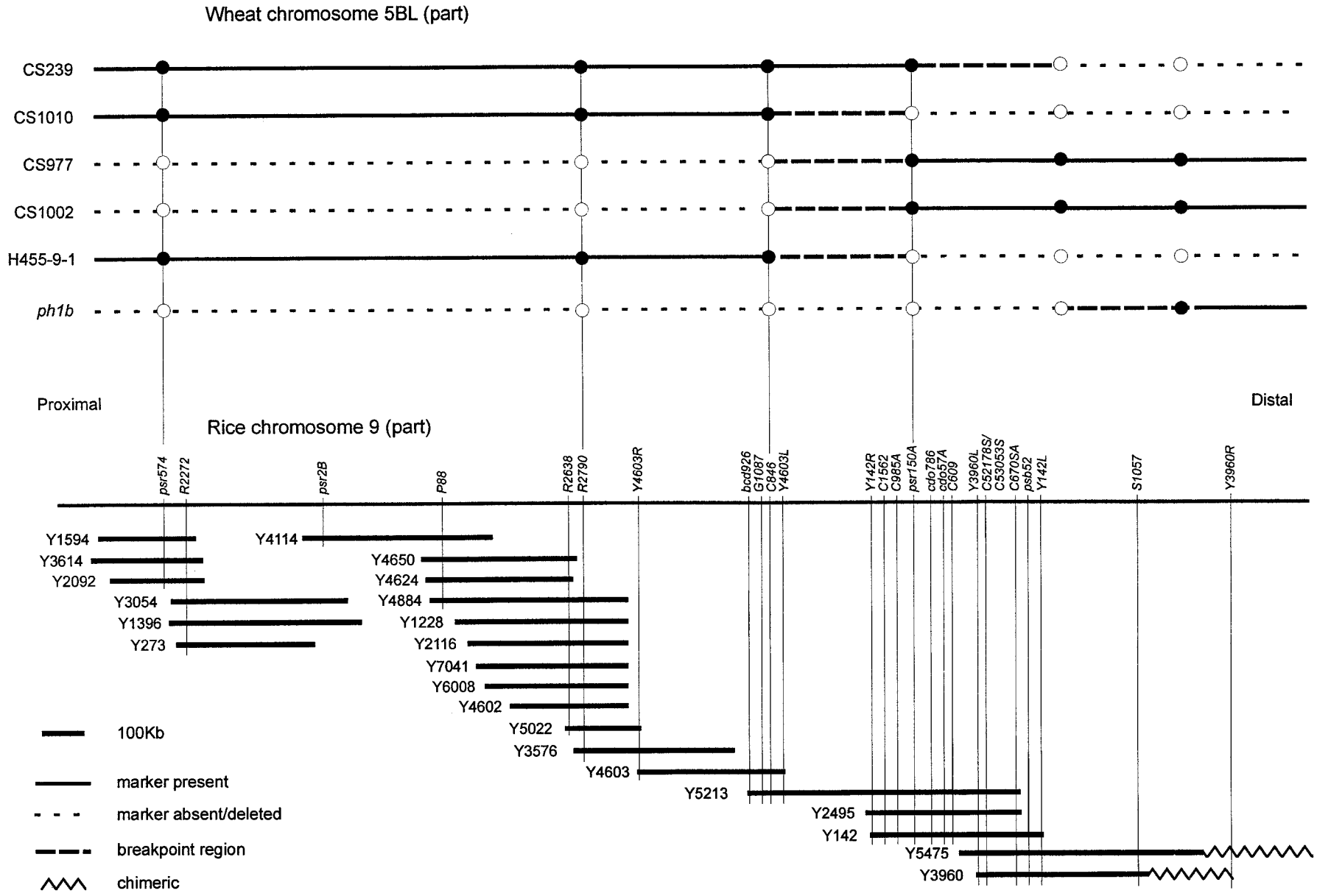

Figure 3.—A comparison of the physical map of rice chromosome *9* with the presence or absence of markers on chromosome *5B* in the new deletion lines. All the lines except CS239 have high homeologous pairing. The marker names are coded according to the species of origin and are designated as follows: *C* (or *Xrgc* in wheat), cDNA clones derived from rice callus library; *R* (or *Xrgr* in wheat), cDNA clones derived from rice root library; *S*, cDNA clones derived from rice shoot tissue; *G* (or *Xrgg* in wheat), rice genomic clones; *psr* (or *Xpsr* in wheat), wheat DNA clones; *bcd*, barley cDNA clones; *cdo*, oat cDNA clones. YACs are designated *Y*****; probes derived from YAC end clones are designated as *Y****R* (right arm) or *L* (left arm). The physical map shows only those markers hybridizing to overlapping YACs.

Comparative physical mapping in rice: Previous analyses indicated that the *ph1b* line carries a deletion of a 70-Mb segment containing the *Ph1* locus on the long arm of chromosome *5B* (Gill *et al.* 1993). Comparative mapping of markers derived from this segment revealed that they were in the same order as related markers on rice chromosome *9*, suggesting a region of gene synteny (Foote *et al.* 1997). A partial YAC contig of this region of rice chromosome *9* was therefore established (Foote *et al.* 1997). Sizing of the rice YAC inserts indicated that the equivalent region to the 70-Mb wheat segment on rice chromosome *9* was at least 4 Mb in size. The analysis of the new deletion lines identified in the present study indicates that the position of the gene(s) controlling the euploid-like pairing lies in the interval defined by the loci *Xrgc846* and *Xpsr150A*. Completion of the YAC contig for the region encompassing these markers would provide a source of more markers and sequence information with which to further characterize the new deletion lines and define the position of the *Ph1* locus.

The rice YACs previously identified by C846, PSR150, and their flanking markers (Foote *et al.* 1997) formed the basis for further analysis in this study. Ends of the left and/or right arms from 10 YACs were cloned using an inverse PCR method (see materials and methods) to generate probes for identifying any overlaps between the YACs. Comparison of the sequences from the resulting YAC ends revealed that 39% matched sequences in the GenBank/EMBL databases. Filters dotted with 47 YACs derived from the part of rice chromosome *9* with apparent gene synteny to the *ph1b* segment of chromosome *5B* were probed with the YAC end clones. Left and right arms of three rice YACs (Y142, Y4603, and Y3960) proved useful for completing a contig from markers *psr2B* to *S1057*, including the region defined by markers *C846* and *psr150A* (Figure 3). The right end arm of Y142 had a significant match (Blastn score, 202; probability, $P = 4e - 50$) with the rice heat shock protein 82 (accession no. Z11920) while its left arm had a significant match (Blastn score, 248; probability $P = 1.9e - 10$) with Arabidopsis ethylene-insensitive-like 3 (accession no. AF004215). The end sequences of the YACs Y4603 and Y3960 had no significant matches in the GenBank/EMBL databases. Southern blot analysis reveals that, in wheat and rice, the probes CDO786, PSR150, and CDO57 all detect a banding pattern similar to that detected by the rice heat-shock protein 82 probes C985 and R1562 (Kurata *et al.* 1994). Sequencing of the probe PSR150 revealed a significant match (Blastn score, 379; probability $P = e - 103$) with the rice heat-shock protein 82. Moreover, two heat-shock protein 82 loci are located on rice chromosome *9* in similar arrangement with other markers to the two corresponding heat-shock protein loci (*Xpsr150A* and *Xpsr150B*) on wheat chromosome group *5* (Foote *et al.* 1997). As described above, one of these loci (*Xpsr150A*) is located within the *ph1b* and *ph1c* deletions and the other locus (*Xpsr150B*) is outside both deletions. The interval between the rice heat-shock protein locus corresponding to *Xpsr150A* and *C846* is no more than 400 kb in the rice genome (Figure 3). Shotgun sequencing of the entire region of the rice genome defined by these two markers is currently underway using subclones from rice bacterial artificial chromosomes. The sequence data will enable the deletion breakpoints (lines CS977, CS1002, CS1010, and H455-9-1) that occur between loci *Xrgc846* and *Xpsr150A* to be characterized, thereby further delimiting the *Ph1* locus.

In this study, the effect of the *Ph1* locus on homologous/homeologous pairing as observed at metaphase I has been scored. However, the *Ph1* locus has been shown to affect the association of homologues via centromere interactions during premeiotic floral development, the structure of the centromeres, the timing of telomere bouquet formation and telomere pairing, as well as recombination between homeologous chromosomes (Luo *et al.* 1996; Aragon-Alcaide *et al.* 1997a; Martinez-Perez *et al.* 1999). Chromosome pairing of the new deletion lines of the *Ph1* locus will be analyzed in detail using anther sectioning and confocal microscopy to determine whether all these effects can be defined to the same region or different regions lying between the *Xrgc846* and *Xpsr150A* loci. Combining this information with deletion breakpoint analysis of the *Ph1* mutants will reveal whether the *Ph1* locus is a single gene, a multigene family, or a complex of linked genes affecting different premeiotic and meiotic processes.

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